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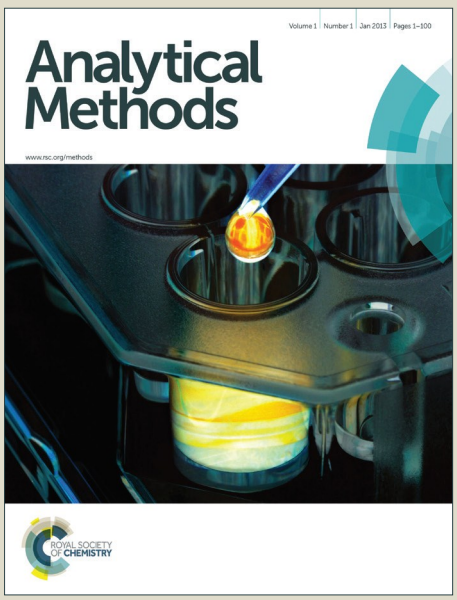


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PAPER

# ICP-MS DNA Assay based on Lanthanide Labels and Hybridization Chain Reaction Amplification

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A novel ICP MS DNA assay based on lanthanide labels and hybridization chain reaction amplification has been developed. This approach utilizes an enzyme-free amplification and can operate in mild conditions. The developed assay achieves desirable sensitivity and is suitable for multiple DNA targets analysis because a general DNA hybridization approach is applied to capture target DNA. Considering lanthanide labels and ICP-MS have excellent ability in high-level multiplexing analysis, this approach holds great potential for practical use in quantitative determination of DNA targets for future clinical applications.

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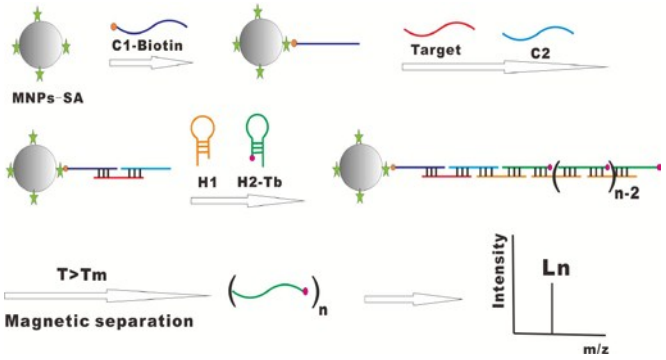
## 1 Introduction

Quantification of nucleic acids is important for biological research and clinic diagnostics. Due to the low abundance of nucleic acids in many biological samples, it is crucial to develop amplification methods to enable sensitive and selective detection of DNA samples. Conventionally well-established technique for nucleic acid assay such as northern blotting technology [1] and DNA microarrays [2] without amplification step might hinder their applications in nucleic acid clinical diagnostics. Hence, various kinds of nucleic acid amplification methods have been developed to improve the sensitivity.[3-9] Fluorescent labeling approach has been widely used in these methods for signal readout due to its sensitivity and easy labeling process. Nevertheless, when it comes to multiplex analysis, the fluorescent labeling method often suffers from photo bleaching, spectral overlap and the lacking of ability for high-level multiplex targets analysis. Inductively-coupled plasma-mass spectrometry (ICP-MS) is a very sensitive technique for element analysis and has become a common practical analysis method recently. [10-12] Hence, by introducing tags such as metal nanoparticles (gold or silver), or lanthanide element for ICP-MS measurement, various biosensing strategies for biomolecules have been developed. [13-18] Moreover, ICP-MS has very high resolution based on different mass to charge ratio, which makes it quite suitable for multiplex analysis. Lanthanide ions are small, stable, heat-resistant labels and are rare in biological samples. Also these ions have very similar properties and can be tagged to biomolecules with same methods, which favors multiplex analysis. Owing to these excellent properties, lanthanide ions as a novel element label coupled with ICP-MS detection technique for biomolecules analysis has gain great attention in recent years. In a recent work, simultaneous quantifications of 15 DNA targets using 15 different lanthanide ions as labels has been achieved by employing a classic sandwich configuration with capture DNA probe, target DNA and lanthanide labeled report DNA probe. [19] This work indicates the excellent ability of ICP-MS technique for high-level multiplexing analysis. To further improve the detection sensitivity, rolling cycle amplification [20] and ligation meditated amplification [21] combined with lanthanide tag using ICP-MS as a detection technique for nucleic acid assay have been developed shortly after.

Herein, we report for the first time ICP-MS coupled with hybridization chain reaction (HCR) amplification technique for nucleic acid detection. Unlike most nucleic acid amplification methods which require enzyme mediation, HCR is an enzyme-free amplification method where a hybridization event is triggered by a DNA sequence and leads to the polymerization of two hairpin oligonucleotides (H1 and H2) into a long nicked dsDNA molecule. [22-23] Moreover, HCR is an isothermal process and operates in mild conditions. The simplest HCR system requires an initiation DNA sequence and two hairpin species of which the potential energy needs to match. In our strategy, a general sandwich structure is employed to capture target DNA and initiate HCR amplification where one of the hairpin probe-H2 is labeled with lanthanide ion (H2-Ln) for ICP-MS detection. The lanthanide ion is labeled onto oligonucleotide by the use of 1,4,7,10-tetraazacyclododecane-1,4,7-tris-aceticacid-10-maleimidoethylacetamide (MMA-DOTA).[24-26] (Figure S1 ESI†). As shown in scheme 1, streptavidin (SA) conjugated MBs is employed to immobilize biotinylated capture DNA probe 1 via the specific biotin-streptavidin interaction. Target DNA, capture DNA probe 1, and capture DNA probe 2 forms a sandwich construction because of the partially complementary sequence. Besides the complementary sequence for target DNA, the capture DNA probe 2 also contains an initiating sequence to trigger a chain reaction of the two hairpin species H1 and H2-Ln to form a nicked double helix. Hence, each target DNA

molecule amplifies the signal by forming the double helix with hundreds to thousands H2-Ln molecules. After magnetic separation process for removing excess DNA probes, the H2-Ln can be released from the magnetic beads into the solution via a simple thermal process and the ICP-MS signal from the solution is correlated with target DNA concentration. This method utilizes DNA hybridization to capture target DNA and can be easily adapted for multiplex DNA targets detection using the same initiator, H1 and H2 sequences system. By altering sequences of capture DNA probe 1 and capture DNA probe 2, as well as lanthanide ion tags. Different DNA targets can be detected simultaneously.




**Scheme 1.**Illustration of ICP-MS detection of DNA based on lanthanide labels and hybridization chain reaction amplification.

## 2 Experimental

### 2.1 Reagents and chemicals

Streptavidin-coated magnetic beads M-280 were obtained from New England BioLabs (NEB, Ipswich, MA). 1, 4, 7, 10-tetraazacyclododecane-1,4,7-trisaceticacid-10-maleimidoethylacetamide (MMA-DOTA) was purchased from Macrocyclics (Dallas, TX) and used for loading lanthanide ions. $TbCl_3{\cdot}6H_2O$, tris (2-carboxyethyl) phosphine (TCEP) were purchased from Sigma-Aldrich (Saint Louis, Missouri). Reaction buffer (50 mM sodium phosphate buffer containing 500 mM NaCl and 50 mM $MgCl_2$, pH=6.8) was used for the HCR reaction. Hybridization buffer (H-buffer, 20 mM Tris-HCl +750 mM NaCl + 5 mM KCl + 5 mM $MgCl_2$) and washing buffer (W-buffer, 7 mM Tris-HCl + 150 mM NaCl + 50 mM KCl + 5 mM $MgCl_2$ + 0.05% Tween-20) were used for DNA hybridization and magnetic beads washing, respectively. Ultrapure water was prepared by a Millipore water purification system (≥18 MΩ cm resistivity, Milli-Q, Millipore) and used throughout the experiments. All the oligonucleotides designed in this study were synthesized and purified by Sangon Biotech Company, Ltd. (Shanghai, China) in HPLC quality(Table S1, ESI†).

### 2.2 Labeling H2 with Lanthanide ion

Terbium ion-coded DNA (H2-Tb) was prepared according to previous reported articles. First, 2 nmol of sulfhydryl-terminated H2 was dissolved in 150 μL 100 mM $NH_4Ac$ (pH 6.8), then 10 μL of 2 mM freshly prepared TCEP (10 fold molar excess) was added into the solution which was kept at 37 °C for 1 h in order to open the possible -S-S- bonds and to ensure that the sulfhydryl groups remain in the form of -SH. Afterwards, 40 μL of 5 mM MMA-DOTA (100 fold in excess compared to H2) was added to allow the reaction between the SH group of the H2 and the maleimide group of MMA-DOTA at 37 °C for 1 h to obtain H2-MMA-DOTA. Tb was loaded into the DOTA moiety at pH 5.8 (adjusted by 1M HCl and maintained with 100 mM $NH_4Ac$ (pH 5.8) for 1 h at 37 °C by adding 30 μL of 50 mM $TbCl_3$. The H2-MMA-DOTA-Tb (H2-Tb) was purified by reverse phase HPLC separation according to previous literature [20]. An Agilent HPLC system equipped with eclipse XDB-C18 column (250 mm in length, 4.6 mm i.d., and particle size 5 μm) were used for oligonucleotide purification. A gradient elution program was employed for purification. Firstly, the mobile phase A (0.1M triethylammonium acetate solution, pH 7.4) was maintained for 4 minutes to elute extra amounts of DOTA-Ln and TCEP, then the mobile phase B ( 5% triethylammonium actate in acetonitrile) was increased from 10% to 22% in 20 min for eluting then H2-Tb at a flow rate of 1.0 mL $min^{-1}$. The H2-Ln powder obtained via freezing drying was redissolved with distilled water to the concentration of ~10 μM estimated by UV absorbance experiments for further use. The ICP-MS measurement was employed for confirmation of the successful linkage of H2-Tb. The concentration of H2-Ln products was estimated based on the calibration curve of standard $TbCl_3$ solutions (Figure S2, ESI†). By diluting the dissolved product solution to make the signal in the calibration curve range, the concentration of the product was determined to be ~10 μM, which is consistent with the UV absorbance measurements.

### 2.3 Gel electrophoresis performance

HCR reactions were performed in the solution without using SA-MBs. Capture DNA probe 2 was used for triggering the chain reaction. Different concentrations of capture DNA probe 2 were used for HCR reactions. Each sample was incubated in reaction buffer at 37 ºC for 2 h. The resultant mixture was collected and analyzed using gel electrophoresis in 4 % (w/w) agarose stained by 0.5 μg/mL goldview and 0.5 μg/mL ethidium bromide. Electrophoresis was performed at a constant voltage of 101 V for 120 min with a load of 10 μL of sample in each lane. The gel was visualized using a Bio-RAD ChemiDox XRS gel imaging system (Bio-RAD technology Company, America).

### 2.4 HCR based assay

Biotinylated capture DNA probe 1 (50 μL, 100 μM) was incubated with streptavidin-coated magnetic beads(SA-MBs, 2 μL, ~$1x10^9$ particles/mL ) for 1 h at 25 °C with gentle shaking and then washed with W-buffer. The SA-MBs were then incubated with a blocking DNA strand (5'-TTTTTT-biotin) at 25 °C for 30 min to block undesired nonspecific absorption

sites on the SA-MBs surface. The as prepared SA-MBs-C1 can be stored at 4°C until use. A series of target DNA solutions with different concentration were incubated with SA-MBs-C1 and capture DNA probe 2 in the H-buffer at 25 °C for 30 min, then H1 (10 μL, 10 μM) and H2-Ln (10 μL, 10 μM) were added and incubate with reaction buffer for 2 h at 37℃. Magnetic separation was performed to retain the SA-MBs and remove the excess DNA probes. The H2-Ln was then released into solution from the SA-MBs surface by heating at 90 °C for 20 min and cooling rapidly. The supernatant was analyzed using ICP-MS instrument. Samples in bovine serum matrices were also determined using this procedure.

### 2.5 ICP-MS detection

Lanthanides were quantified using ICP-MS instrument (PerkinElmer NexION 300, Canada).The optimizing of ICP-MS operational parameters are shown in supporting information(Table S2, ESI†).

## 3 Results and discussion

For demonstration, Tb ion was selected as the label and a DNA sequence partially from human thymidine kinase 1 mRNA (TK1mRNA) was chosen as the target DNA. The experiment condition in section 2 was chosen based on previous literature and optimization experiments. High salt concentration was used since it favors HCR reaction. The 2 h reaction time was decided based on the ICP-MS signal intensities obtained by using 500 pM target to perform assay with different reaction time. (Fig S3, ESI†). To confirm the feasibility of the approach, MS signal of different reaction conditions are recorded. As shown in Fig. 1, buffer solution and 10% bovine serum solution show barely MS signal which is consistent with the extremely low background of lanthanide element in environmental and biological samples. The samples were performed in the buffer solutions according to the procedures described in section 2.4. After magnetic separation, thermal release and resuspention process, the upper solution was sent for ICP-MS measurement where MS intensity of $^{159}$Tb was recorded. When incubating the SA-MBs with capture DNA probe 1, capture DNA probe 2, H1 and H2-Ln, a very small MS signal was obtained for the sample probably due to the weakly non-specific adsorption of the HCR products or excess H2-Ln onto the magnetic beads. In contrast, incubation of magnetic beads with capture DNA probe 1, capture DNA 2, H1 and H2-Ln in the presence of 200 pM target DNA displayed a strong MS intensity signal, indicating the HCR products were conjugated onto the SA-MBs via hybridization of capture DNA probe 1, capture DNA probe 2 and target DNA. Two control experiments using different DNA sequences other than target DNA (S1, S2 each at 2 nM) did not show an increased MS intensity comparing with that in the absence of target DNA. This testified the capture DNA probe1 and capture DNA probe 2 were specific to target DNA sequence; and this approach has excellent selectivity for DNA analysis.

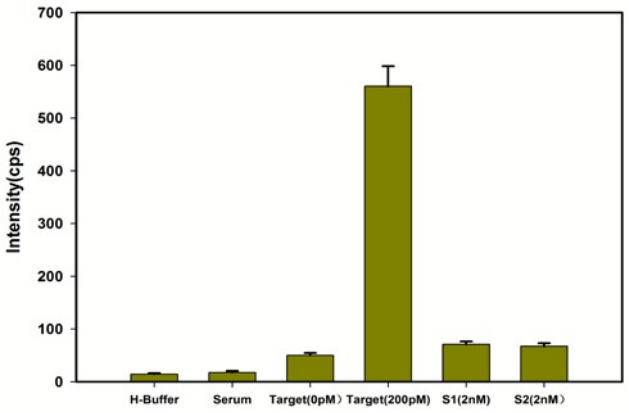



**Fig.1** MS intensity responses for HCR based assay under different conditions. From left to right: reaction buffer; 10% bovine serum; C1+C2+H1+H2-Ln; C1+C2+H1+H2-Ln+target DNA; C1+C2+H1+H2-Ln+S1;C1+C2+H1+H2-Ln+S2, respectively. Error bars are standard deviation of three repetitive experiments.

Agarosegel electrophoresis analysis was performed to verify the mechanism of HCR between capture DNA probe 2 and the hairpin probes (H1 and H2-Ln). The experiments were performed in solution without using of SA-MBs. As illustrated in Fig.2, the band position in lane 4 is very similar with H1 (lane 2) and H2 (lane 3) indicates no HCR occurs when mixing H1 and H2-Ln without capture probe 2. On the other hand, smears appeared in the 5th, 6th and 7th lanes when different concentrations of capture DNA probe 2 were mixed with H1 and H2-Ln, indicating HCR had occurred and formed long-nicked DNA polymers in the presence of initiation sequence. In the HCR system, amplification of the initiator recognition event continued until the supply of H1 or H2 was exhausted, and the molecular weight of the resulting polymers was not an exact numerical value, but with different length of products in an approximate range. Therefore, the resulting polymers in lane 5, 6, and 7 appear as smears instead of bands, which is consistent with other research involved with HCR amplification.[22,27-30]

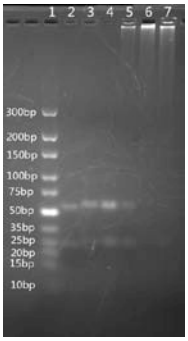



**Fig .2** Agarose gel electrophoresis images of HCR under different conditions. Lane 1, DNA marker (10-300bp); Lane 2, H1 (500 nM); Lane 3, H2-Ln (500 nM); Lane 4, H1 (500 nM)+ H2-Ln (500 nM); Lane 5, H1 (500 nM)+H2-Tb (500 nM) +C2 (100 nM); Lane 6: H1(500 nM)+H2-Tb (500 nM) +C2 (250 nM); Lane 7: H1 (500 nM)+H2-Tb (500 nM) +C2 (500 nM).

The ability of quantitative analysis of target DNA was investigated by performing a series of samples with different target DNA concentrations. Fig.3 shows a dynamic correlation between MS intensity and target DNA concentration in the range from 1pM to 1nM. The inset reveals a linear correlation between the MS intensity and the logarithm of target DNA concentration (y = 112.69ln(x) - 54.842, $R^2$ = 0.9466).The detection limit is estimate to be 300 fM according to 3σ rule. The results demonstrated signal enhancement compared to amplification-free ICP-MS DNA assay[19]. The detection limit and range is comparable with previous reported rolling circle amplification (RCA) and ligase chain reaction based ICP-MS DNA assay [20-21]. This is consistent with the several hundred to several thousand-fold signal enhancement normally provided by these linear amplification approaches. While comparing with the fluorescent dye labeled HCR DNA assay [30], this ICP-MS based HCR DNA assay did not show improvement in sensitivity. This is probably due to the relatively low HCR efficiency occurred in magnetic bead surface instead of homogenous solution. The reported RCA based ICP-MS DNA showed only 190-fold signal enhancement also indicates relative low amplification efficiency. However, the ICP-MS based DNA assay is capable of quantifying many different ions simultaneously, comparing with fluorescent labelling approach.

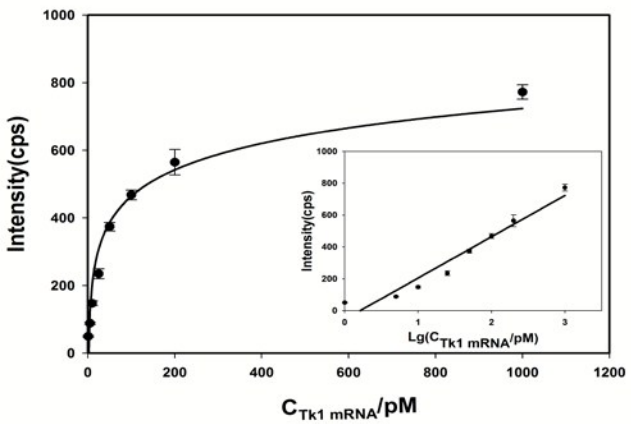



**Fig. 4** HCR based assay in response to TK1 mRNA targets of various concentration. Error bars are standard deviation of three repetitive experiments.

To evaluate the assay in complex biological media, the detection of TK1 mRNA in 10% bovine serum samples was also performed. The results revealed that the MS signal responses obtained in the serum samples were in good consistency with those obtained in buffer solutions (Figure S4, ESI†). Four bovine serum samples with added TK1 mRNA of different concentrations were measured using this approach and satisfactory recoveries between 91.2% and 105.4% were obtained (Table S3, ESI†), indicating the potential this strategy for real sample analysis.

## 4 Conclusion

We have reported a novel ICP-MS DNA assay using lanthanide ion as label and coupled with HCR amplification. The HCR amplification is an excellent isothermal amplification approach which requires no enzyme and can operate under mild conditions. Our strategy describes a general platform of HCR amplification for DNA analysis. As discussed above, multiple DNA targets can be detected simultaneously using this biosensing strategy by altering capture DNA probes for specific DNA targets; and lanthanide ions label enables the high resolution for the ICP-MS based multiplexing analysis. Therefore, considering the importance of multiple biomarkers for disease diagnosis, the developed assay can be useful for practical clinic diagnostics due to the excellent high-level multiplexing analysis ability.

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## Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental details and additional Figures. See DOI: 10.1039/b000000x/

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